

Discrete extraction of the Ca atom functional for O₂ evolution in higher plant photosystem II by a simple low pH treatment

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Treatment of spinach PS II membranes with a citrate solution at pH 3.0 totally inactivated O₂ evolution concomitant with a 50% decrease in Ca abundance. Notably, neither the abundance of Mn and extrinsic proteins nor the activity of DPC photooxidation was at all affected by the treatment. The treated membranes evolved O₂ at a high rate in the presence of exogenous Ca²⁺, but the activity was sensitive to EDTA. However, when the treated membranes were incubated with Ca²⁺ for a few tens of minutes, the O₂-evolving activity became EDTA-resistant, suggesting a firm re-ligation of Ca²⁺ to the Ca-binding site. It was indicated that spinach PS II contains two Ca atoms per reaction center, and that the low pH citrate treatment selectively removes one of the two Ca atoms that is specifically functional for O₂ evolution, even in the presence of all three extrinsic proteins.

Oxygen evolution; Photosystem II membrane; Ca²⁺ extraction; Low pH treatment; 16 kDa protein; 24 kDa protein; 33 kDa protein

1. INTRODUCTION

Ca is a cofactor involved in PS II reactions [1,2]. In higher plants, exogenous Ca²⁺ stimulates O₂ evolution by NaCl-washed [3,4] and CaCl₂-washed [5,6] PS II membranes, both of which are devoid of the 24 and 16 kDa extrinsic proteins (former) and the 33, 24 and 16 kDa extrinsic proteins (latter). Similar Ca²⁺-dependent stimulation of O₂ evolution has been reported for the O₂-evolving PS II reaction center complex which retains Mn and only one extrinsic protein (33 kDa) [7]. As the demand for exogenous Ca²⁺ is manifested mostly in salt-washed PS II, it has been proposed that an extrinsic protein (24 kDa) affords a high-affinity binding site for Ca [3]. However, there are some

reports that the release of Ca from salt-washed PS II membranes requires light [6,8], suggesting a Ca-binding site not on the extrinsic protein but on the core of the PS II reaction center.

As to the functional site of Ca in PS II reactions, it has been considered that Ca functions either in S-state transitions [9] or in electron transport around the reaction center [8]. Based on the effects of Ca removal on the oscillation pattern of O₂ flash yield and thermoluminescence, we proposed that Ca functions in both of the above-mentioned sites [6]. However, since the extents of inhibition elicited by Ca removal were not total but partial, unambiguous confirmation of these views remained difficult.

In cyanobacterial PS II preparations, on the other hand, it has been unambiguously shown that the reaction center contains only one Ca atom which functions in the electron transfer between Z and P680⁺ [10-12], although it remains unclear whether another Ca is required for S-state transitions in cyanobacterial systems.

Recently, a precise determination of Ca in higher plant PS II has been reported by Cam-

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Abbreviations: PS, photosystem; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

marata and Cheniae [13]. They treated PS II membranes with a high concentration of NaCl containing a chelator and ionophore and found that the treated membranes contained about one Ca per reaction center and showed O₂ evolution strongly dependent on exogenous Ca²⁺. However, the restored O₂-evolving activity was inevitably affected by the absence of extrinsic proteins, and it appears that some ambiguities still remain as to the relationship between Ca abundance and activity.

Under these circumstances, a technique for discrete extraction of Ca without affecting the extrinsic proteins is desired. Here we report a simple technique, citrate treatment at pH 3.0, which selectively removes one of the two Ca atoms per reaction center in spinach PS II. The extracted membranes totally lose O₂ evolution and the lost activity is restored close to 80% on re-insertion of Ca²⁺ into the Ca-binding site.

2. MATERIALS AND METHODS

BBY-type O₂-evolving PS II membranes [14] were prepared from spinach with modifications as described in [6]. The PS II membranes were washed with 400 mM sucrose, 20 mM NaCl (pH 6.5), and resuspended in the same non-buffered medium. The membranes were then treated with 400 mM sucrose, 20 mM NaCl, 10 mM citrate NaOH (pH 3.0) at 2 mg Chl/ml. After 5 min incubation at 0°C in the dark, the treated membranes (denoted here as low pH-treated membranes) were diluted with 400 mM sucrose, 20 mM NaCl and 40 mM Mes-NaOH (pH 6.5) to adjust the final pH to around pH 6.5, and then subjected to electron transport measurements after dilution with the same buffered medium.

For determination of Ca abundance, control and low pH-treated membranes were washed with 400 mM sucrose, 20 mM NaCl, 0.1 mM EDTA·2Na and 40 mM Mes-NaOH (pH 6.5), and then finally suspended in 400 mM sucrose, 20 mM NaCl and 40 mM Mes-NaOH (pH 6.5) at 0.2–2 mg Chl/ml. The resulting membrane suspensions were diluted with 1–10 vols of the same buffer medium, and then finally diluted with 9 vols water which had been deionized and filtered through a Milli Q (Amicon) system followed by treatment with Chelex 100, and Ca and Mn were determined with an atomic absorption spectrophotometer (Shimadzu, 640-13) equipped with a graphite furnace atomizer (GFA-3). Estimation of Ca abundance was made carefully based on the coefficient determined for a linear relation observed between Chl concentration and Ca abundance. All plastic ware used had been carefully washed with 0.1 mM EDTA·2Na solution and rinsed with the above-mentioned purified water.

Protein composition was analyzed by SDS-PAGE as in [5]. O₂ evolution and DCIP photoreduction were measured at 25°C as in [15], being supplemented with salts where indicated. Unless otherwise noted, O₂ evolution was measured with 0.8 mM phenyl-*p*-benzoquinone as electron acceptor.

3. RESULTS

Fig.1 shows the pH dependence of O₂ evolution and its inactivation, in which PS II membranes were treated at various low pH values, the activity being measured in the same medium or after transfer to one of pH 6.5. The O₂ evolution decreased at low pH values and almost completely disappeared at pH 4.0. Over the pH range 6.0–4.5, the inhibited O₂ evolution was reversibly restored when the pH was readjusted to the normal value of 6.5, but the reversibility declined steeply below pH 4.5. At pH 3.0, O₂ evolution was completely inactivated with no restoration at pH 6.5. The pH required to effect half-irreversible inactivation was found to be around 4.0, 1 pH unit lower than that for pH-dependent O₂ evolution. The inset to fig.1 shows the course of irreversible inactivation of O₂ evolution during treatment with a citrate buffer at pH 3.0. The activity declined exponentially according to first-order kinetics and almost totally vanished after incubation for 5 min.

Table 1 lists the effect of low pH treatment on PS II reactions and the abundance of Mn and Ca. In treated membranes, the electron transport reaction with water as electron donor was markedly inactivated while that with DPC as donor was little affected. This indicates that the treatment specifically inactivates the water-oxidation site of PS II. The resulting inactivation is not ascribed to structural disruption of the Mn center (e.g. Mn release), since the treated membranes retained about 4 Mn/220 Chl. In addition, the inactivation is not ascribed to a loss of proteins from the membranes. As shown in fig.2, treated membranes showed the same protein composition in both densitograms (A) and electrophoretograms (B), exhibiting clear retention of the 24 and 16 kDa extrinsic proteins. In contrast to the rather constant values of Mn abundance and protein composition, the abundance of Ca was found to be significantly decreased in treated membranes: 1.9 Ca/220 Chl in control membranes vs 1 Ca/220 Chl. The result suggests that the low pH treatment releases one of the two Ca atoms per reaction center and thereby inactivates O₂ evolution with no effect on the other PS II reactions.

Table 2 details the restoration effect of several divalent cations on O₂ evolution in low pH-treated PS II membranes. The inhibited activity was

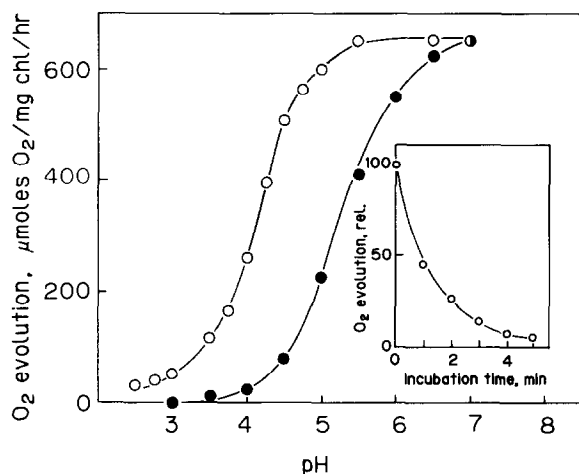


Fig. 1. Dependence on pH of O_2 evolution and its irreversible inactivation. O_2 -evolving PS II membranes were suspended in 40 mM of either citrate (pH 2.5–4.5) or Mes (pH 5.0–6.5) buffer with supplement of 400 mM sucrose and 20 mM NaCl and incubated at 0°C in the dark. After 5 min incubation the activity was measured in the same buffer medium at various pH values (●) or after transfer to the Mes buffer medium at a constant pH 6.5 (○). The inset shows the course of irreversible inactivation of O_2 evolution during incubation at pH 3.0. O_2 evolution was measured with 2,5-dimethylquinone as electron acceptor.

restored up to 76% when 50 mM CaCl_2 was included during both preincubation (10 min) and subsequent activity measurement. Such a restoration effect was pronounced with CaCl_2 , appreciable with SrCl_2 and negligible with MgCl_2 and BaCl_2 . NaCl at 200 mM did not show any restoration effect. About 70% of the activity restored after 10 min preincubation with Ca^{2+} was shown to persist even though Ca^{2+} was eliminated from the assay medium by dilution with an EDTA-containing medium. The survival rate in the presence of EDTA increased to 80–90%, when the duration of preincubation with CaCl_2 was in-

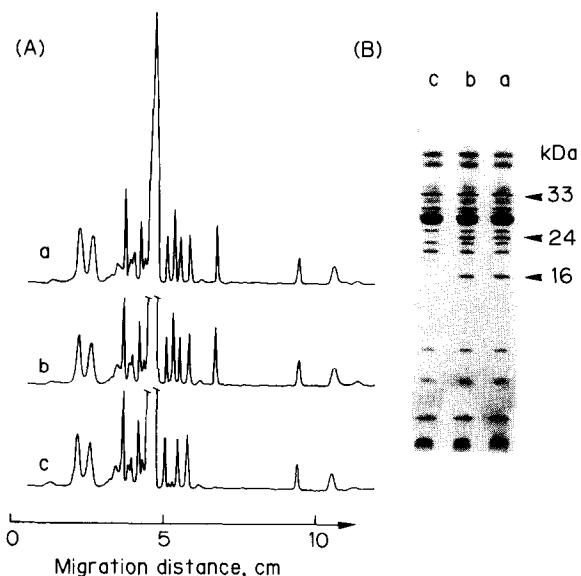


Fig. 2. SDS-PAGE densitogram (A) and electrophoretogram (B) of PS II membranes: (a) control membranes, (b) pH 3.0-treated membranes, (c) 2 M NaCl-washed membranes.

creased to 40 min (not shown). In view of the fact that neither Ca^{2+} nor chelating agents affect O_2 evolution in control membranes (not shown), the result suggests that during the preincubation, the Ca atom is reincorporated and ligated to the proper site in the O_2 -evolving center to acquire EDTA resistance and reconstitute O_2 evolution.

Fig. 3 shows the time course of reconstitution of EDTA-resistant O_2 evolution. The low pH-treated membranes were incubated with 50 mM CaCl_2 at 0°C in darkness for various periods, and then the O_2 -evolving activity was determined in the presence of excess EDTA: final concentrations of Ca^{2+} and EDTA in the assay medium were 1.0 and 2 mM, respectively. The EDTA-resistant activity developed rapidly and reached saturation after 40

Table 1

Effect of low pH treatment on O_2 evolution, DCIP photoreduction, and Ca and Mn abundance

PS II membranes	Electron transport activity			Metal abundance (atoms/220 Chl)	
	$\text{H}_2\text{O} \rightarrow \text{phenyl-}p\text{-benzoquinone}$	$\text{H}_2\text{O} \rightarrow \text{DCIP}$	$\text{H}_2\text{O} \rightarrow \text{DCIP DPC}$	Mn	Ca
	($\mu\text{mol } O_2/\text{mg Chl per h}$)	($\mu\text{mol DCIP}/\text{mg Chl per h}$)	($\mu\text{mol DCIP}/\text{mg Chl per h}$)		
Control	690	590	600	4.2	1.9
Low pH treated	50	90	560	4.0	1.0

Table 2

Restoration of O₂ evolution in low pH-treated membranes after 10 min incubation with various cations at 0°C in darkness

Conditions	O ₂ evolution
	($\mu\text{mol O}_2/\text{mg Chl per h}$) (relative)
Control membranes	540 (100)
Low pH-treated membranes	
No addition	50 (9)
+ CaCl ₂ (50 mM) ^a	410 (76)
+ SrCl ₂ (50 mM)	150 (28)
+ MgCl ₂ (50 mM)	60 (11)
+ BaCl ₂ (50 mM)	30 (6)
+ NaCl (100 mM)	50 (9)
+ CaCl ₂ (50 mM) ^b → CaCl ₂ (1 mM) EDTA (2 mM)	270 (50)
+ CaCl ₂ (1 mM) and EDTA (2 mM) ^c	40 (7)

^a Cations were included during both preincubation (0°C, 10 min, dark), and subsequent activity measurement

^b Treated membranes were preincubated with 50 mM CaCl₂ (0°C, 10 min, dark), and then diluted with an assay medium containing 2 mM EDTA·2Na (final concentration of Ca²⁺, 1 mM)

^c Treated membranes were preincubated with 1 mM CaCl₂ and 2 mM EDTA·2Na (0°C, 10 min, dark), and then directly subjected to activity assay.

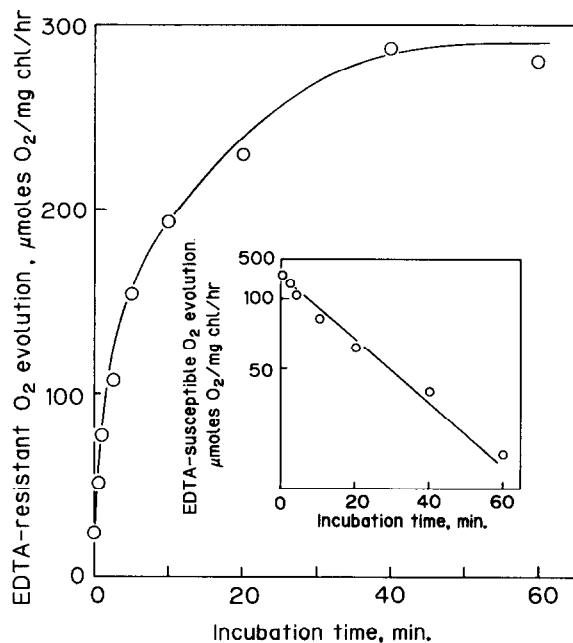


Fig. 3. The course of reconstitution of EDTA-resistant O₂ evolution during incubation with Ca²⁺. Low pH-treated membranes were incubated with 50 mM CaCl₂ (0°C, pH 6.5, dark). After the indicated incubation time, the suspension was diluted with the assay medium containing 2 mM EDTA (final Ca²⁺ concentration, 1.0 mM) and O₂ evolution was measured. The inset shows a semilogarithmic plot of the time course.

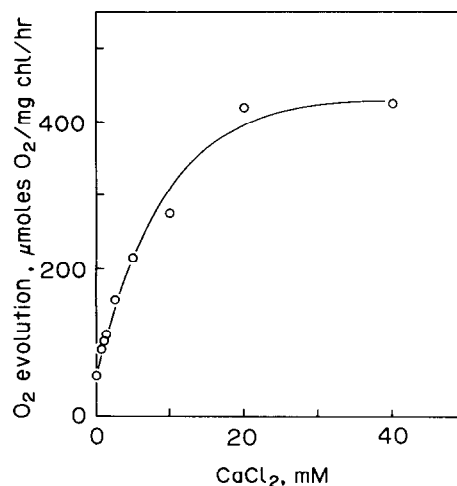


Fig. 4. Dependence on Ca²⁺ concentration of reconstitution of EDTA-resistant O₂ evolution. Low pH-treated membranes were incubated with various concentrations of CaCl₂ (0°C, pH 6.5, dark) for 60 min, and then diluted with the assay medium containing 2 mM EDTA (final concentration of Ca²⁺ was 1.0 mM at maximum after dilution).

min incubation. The inset in fig.3 shows a semi-logarithmic plot of the course of reconstitution, in which the logarithm of the portion of O₂-evolving centers remaining EDTA-susceptible was plotted vs incubation time. The resulting straight line implies that the reconstitution is a first-order process.

Fig.4 shows the dependence on Ca²⁺ concentration of the extent of reconstitution of EDTA-resistant O₂ evolution. The low pH-treated PS II membranes were incubated with various concentrations of CaCl₂ for 60 min followed by activity measurement in an assay medium containing excess EDTA as described in fig.3. The EDTA-resistant O₂ evolution increased with increase in Ca²⁺ concentration to reach a saturation level at about 20 mM with an apparent half-saturating concentration of 5 mM.

4. DISCUSSION

The present results demonstrate that O₂-evolving activity is almost completely inactivated by treatment with a citrate-containing medium at pH 3.0 accompanied by a decrease in Ca abundance from 2 to 1 Ca/220 Chl, while no change occurs in Mn abundance and protein composition. According to the stoichiometry proposed for spinach PS II components, the value of 2 Ca/220 Chl in control membranes is equivalent to 2 Ca atoms per unit of PS II [16]. Thus, our Ca determination is in agreement with the previously reported values for spinach PS II [10,13], although wheat PS II is reported to contain 3 Ca per reaction center [13]. Consequently, as far as spinach PS II is concerned, we may consider that our low pH treatment selectively extracts one of the two Ca atoms per unit of PS II. The pH dependence curve shown in fig.1 suggests that protonation of a carboxyl group is responsible for liberation of the Ca atom from its ligation site. It is worth noting that most Ca-binding proteins commonly possess a structural domain involving an aspartic acid or glutamic acid residue for ligation of the metal [17]. It is thus very probable that a similar domain for Ca binding exists in the PS II protein complex.

From the restoration of O₂ evolution specifically effected by Ca²⁺, we can ascribe the loss of O₂ evolution by the low pH treatment to extraction of one Ca atom from PS II. It is likely that the extracted Ca atom is functional in the O₂-evolving

center, since the electron transport from DPC to DCIP was not affected by the treatment while O₂ evolution was markedly inactivated. It has been reported that O₂-evolving PS II particles from cyanobacteria contain only one Ca atom that is essential for electron transport from Z to P680 [10-12]. Thus, the present results lead us to assume that the acid-extractable Ca atom is functionally different from the Ca atom in cyanobacterial PS II. We may presume in turn that the other Ca atom present in spinach PS II and resistant to low pH extraction affords the electron transport between Z and P680⁺. These considerations are in line with our previous proposal [6] that Ca²⁺ removal inhibits O₂ evolution both by disconnecting the S-system from the reaction center and by blocking the S-state transition.

The concentration of Ca²⁺ required for restoration of O₂ evolution in treated membranes was non-physiologically high, and the process of acquiring EDTA resistance was slow and time dependent. These may suggest that the extractable Ca is not directly involved in water-oxidation chemistry but rather is needed to maintain the structural integrity of the water-oxidation enzyme as required for O₂ evolution. A similar sort of reconstitution of O₂ evolution by exogenous Ca²⁺ has been reported by Ghanotakis et al. [18] to occur when Ca²⁺ is added to NaCl-washed PS II membranes after rebinding of the 16 and 24 kDa extrinsic proteins. They observed that a partially inhibited activity (30-40% of the control) was doubled by Ca²⁺, although their salt-washed membranes still maintained an extremely high abundance of Ca²⁺ [3]. We consider that the rather high background O₂ evolution found after salt washing in their experiments is probably due to incomplete removal of Ca²⁺.

Based on these results and considerations, we conclude that low pH treatment of higher plant (spinach) PS II selectively extracts one Ca atom per unit of PS II, leaving the other Ca atom unaffected. The extractable Ca is essential for functioning of the O₂-evolving system. The low pH treatment technique reported here will be highly advantageous in studying the function of Ca²⁺ in PS II.

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